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METHODS

LC–ESI–MS/MS Identification of Polar Lipids of Two Thermophilic *Anoxybacillus* Bacteria Containing a Unique Lipid Pattern

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Abstract Phospholipids and glycolipids from two recently described species belonging to the thermophilic genus *Anoxybacillus* were analyzed by liquid chromatography–electrospray tandem mass spectrometry (LC/ESI–MS/MS). Analysis of total lipids from the facultatively anaerobic *A. bogrovensis* on a HILIC (Hydrophilic Interaction LIquid Chromatography) column succeeded in separating diacyl- and plasmalogen phospholipids. The LC/ESI–MS/MS analysis of the strict aerobe *A. rupiensis* revealed the presence of different unique polar lipids, predominantly alanyl-, lysyl-, and glucosyl-phosphatidylglycerols and cardiolipins. Each of the classes of polar lipids was then analyzed by means of the ESI–MS/MS and more than 140 molecular species of six lipid classes from *A. bogrovensis* and nearly 200 molecular species of nine classes of polar lipids from *A. rupiensis* were identified. Five classes of unidentified polar lipids were detected in both strains. Plasmalogens were thus determined for the first time in a facultatively anaerobic bacterium, i.e. *A. bogrovensis*.

Keywords *Anoxybacillus* · Plasmalogens · *O*-Aminoacylphosphatidylglycerols · *O*-Aminoacyl cardiolipins · Liquid chromatography–electrospray tandem mass spectrometry · HILIC column

Abbreviations

ACN	Acyl carbon number
APtdGro	Acyl-phosphatidylglycerol
BMP	Bismonoacylglycerophosphate
CID	Collision-induced dissociation
Ptd_2Gro	Cardiolipin or diphosphatidylglycerol, or more precisely 1,3-bis(*sn*-3′-phosphatidyl)-*sn*-glycerol
FA	Fatty acid
FAME	Fatty acid methyl ester
GC–MS	Gas chromatography–mass spectrometry
HILIC	Hydrophilic Interaction LIquid Chromatography
LC/ESI–MS/MS	Liquid chromatography–electrospray ionization tandem mass spectrometry
PlsEtn	Ethanolamine plasmalogen
PlsGro	Glycerol plasmalogen
PlsOH	Plasmalogen phosphatidic acid
PtdEtn	Phosphatidylethanolamine
PtdGro	Phosphatidylglycerol
PtdOH	Phosphatidic acid
TLC	Thin layer chromatography

Introduction

Thermophilic bacteria are type extremophiles that are adapted to life at high temperature (optimum growth above 50 °C). They inhabit separate permanently hot niches such as

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areas with geothermal and volcanic activity. They were among the first inhabitants of Earth and hold the lowest branches in the global phylogenetic tree of life; their study is thus intimately related with the question of the origin of life and biological evolution. Thermophilic adaptation to severe conditions resulted in the development of several unique mechanisms, one of which is a specific composition of microbial membranes, which form the lipid bilayer permeability barrier of cells. The ability of bacteria to control and modify the biophysical properties of their membrane is achieved by a change of phospholipid composition in response to environmental changes. As an adaptation to the fluidizing effects of increased growth temperature, it is common to observe an increase in the proportion of long chain and saturated fatty acids within the membrane [1].

The efforts to identify new lipids, which may extend the scientific knowledge and have potential practical application, include the isolation and study of new microorganisms in long-known genera. The genus *Bacillus* has a long taxonomic history. Since it was described by Cohn in 1872, its systematics has undergone major revisions [2]. Based on the large number of novel endo-spore forming bacteria described in the 1990-s, Zeigler [3] described 15 genera as *Bacillus* sensu lato (*Bacillus*-like group) and predicted the list of approved *Bacillus*-like genera to expand rapidly during the new decade. One of the recently described genera in this clade is the genus *Anoxybacillus*; its representatives are thermophilic and alkaliphilic or alkalitolerant, chemoorganotrophic bacteria. Though representatives of *Anoxybacillus* have been first identified as anaerobes [4], an additional amendment [5] referred to them as aerotolerant anaerobes. Further, most of these species have been found to be facultative anaerobes and even for some species anaerobic growth was registered only in some conditions [6]. The genus comprises currently 15 species [7] isolated from different geographical areas—Russia, Belgium, Turkey, Mongolia, etc. Two of the species, *A. rupiensis* [8] and *A. bogrovensis* [9] were recently isolated from Bulgarian hot springs and novel molecules of lipids including, e.g., plasmalogens were envisaged in these new microorganisms.

Plasmalogens are any derivatives of *sn*-glycero-3-phosphoric acid that contain *O*-alk-1′-enyl residue attached to the glycerol moiety at *sn*-1 position and a polar head made of a nitrogenous base or glycerol moiety. Their occurrence is limited to strictly anaerobic bacteria and they have not yet been identified in aerobic or facultatively anaerobic bacteria. They are common constituents of animal lipids from invertebrates to humans [10] and are found mostly as plasmalogen phosphatidylcholine, plasmalogen ethanolamine (PlsEtn), plasmalogen phosphatidylglycerol (PlsGro), and plasmalogen phosphatidic acid (PlsOH).

Phosphatidylglycerol (PtdGro) and cardiolipin (Ptd_2Gro) are the major anionic membrane lipids in most bacteria [11]. Forms of PtdGro and Ptd_2Gro modified by different amino acids have been described; for instance, lysyl-phosphatidylglycerol (lysyl-PtdGro) and other aminoacyl esters of PtdGro, such as alanyl-PtdGro, or ornithyl-PtdGro, are major embrane lipids in several Gram-positive bacteria (Firmicutes) [12]. Lysyl-PtdGro constitutes a major membrane lipid in different strains of bacteria [13, 14] such as *Bacillus subtilis* [15], *B. anthracis* [16], and *Listeria monocytogenes* [17]. Alanyl-PtdGro was isolated from *Clostridium perfringens* [14]. A large variety of PtdGro-derived lipids are present in *Enterococcus faecalis* (formerly known as *Streptococcus faecalis*) which probably has alanyl-PtdGro, 2′-lysyl-PtdGro, 3′-lysyl-PtdGro, 2′,3′-dilysyl-PtdGro, arginyl-PtdGro, and a diglucosyl derivative of PtdGro [18].

Lysyl-, alanyl-, and glucosyl-Ptd_2Gro have also been described in bacteria [19]. The paper by Peter-Katalinic and Fischer [20], who identified α-D-glucopyranosyl-, D-alanyl- and L-lysylcardiolipin from Gram-positive bacteria (*Streptococcus* sp., *Vagococcus fluvialis* or from *Listeria welshimeri*), can be considered a basic study in the field of mass spectrometry of these polar lipids. A detailed structural study of a cardiolipin and an integral membrane protein was reported for *Rhodobacter sphaeroides* AM260W, which is able to grow heterotrophically via fermentation and aerobic and anaerobic respiration [21].

Identification of these polar lipids is relatively difficult. Many studies using two-dimensional TLC identified only some of the spots while other spots remained unidentified. Other methods of choice are HPLC, especially in combination with mass spectrometry, or mass spectrometry alone, including tandem mass spectrometry [22]. These methods were used for identifying 16 bacterial strains, e.g. *Bacillus subtilis* or *Staphylococcus aureus* [23].

Further, amphiphilic molecules such as phospholipids can be separated on a Hydrophilic Interaction LIquid Chromatography (HILIC) column, but this method has been described in only a few papers, with relatively controversial results concerning especially the elution of individual phospholipid classes [24–26].

This report is part of our investigation of bacterial lipids within the framework of a comprehensive program on the analysis and biosynthesis of lipids [27, 28]. We present here the separation and identification by LC–ESI/MS^2 of intact plasmalogen phospholipids from diacyl phospholipids of the facultatively anaerobic bacterium *A. bogrovensis*, and nearly 200 molecular species of nine classes of polar lipids from *A. rupiensis*.

Results and Discussion

The total lipid extracts prepared from *A. bogrovensis* by the modified Bligh and Dyer method [29] have been

pre-fractionated by using a cartridge with aminopropyl silica-based polar bonded phase, and further separated on a HILIC column into the individual lipid classes [28]. The chromatographic conditions have been optimized to provide the best separation of all lipid classes. The composition of mobile phase is crucial for the separation of lipid classes. Good resolution of lipid classes was obtained with the mixture of hexane/2-propanol/aqueous ammonium acetate [30] but better selectivity, chromatographic resolution and low background noise were observed in a mobile phase containing methanol–acetonitrile–water with ion-pairing agents such as ammonium salts [24]. The concentration of water and salts including the pH value of mobile phase are crucial parameters for separation and identification of ionic lipids. The use of ion-pairing agents (e.g., ammonium acetate or formate) significantly improves the chromatographic resolution and ionization efficiency of lipids. The best separation and signal intensity have been achieved in mobile phases with 1 mM aqueous ammonium acetate at pH 7 [28]. Lipids from *A. bogrovensis* were separated (Fig. 1) and quantified based on the negative-ion ESI mass spectra [Table 1S (Supplement)]. The retention times of eluted lipids are based on their adsorptions (electrostatic forces) on the HILIC column and showed that lipids with higher polarity or stronger dissociation are more retained. Most lipid classes were baseline separated but some lipids were co-eluted and five peaks were not identified (U1–U5); see Fig. 1 and Table 1S (Supplement).

Individual strains of genus *Bacillus* are being continuously reclassified on the basis of 16S rRNA gene sequence analysis [31]. This is the probable reason why older papers about lipids of bacilli describe considerable heterogeneity in the lipid content [32]. On the other hand, it should be noted that the polar lipids of most bacilli have not yet been identified. Most of the authors are satisfied with the identification of 3–4 polar lipids on the basis of matching the R_f of two-dimensional TLC [33, 34]. Other spots on the TLC plate remain unidentified; thus Minana-Galbis et al. [33] described eight spots, Khianngam et al. [35] four spots and Kampfer et al. [36] did not identify four, i.e. half of the total number of spots.

The two species of the genus *Anoxybacillus* that we analyzed differ in the composition of their polar lipids. The major phospholipids present are Ptd_2Gro and PtdGro, which confirms that these species belong to the bacilli [12]. Preliminary papers describing the polar lipid composition of the genus *Anoxybacillus* indicated that the strains contain a large number of unidentified polar lipids [33, 37] and share a similar chemical composition. The chemical composition of our two strains also centers around the presence of three polar lipid classes (Ptd_2Gro, PtdGro, PtdEtn), and the dominance of iso- and anteiso-fatty acids (Table 1; see Ref. [8, 9]). Similar observations on *Anoxybacillus* fatty acids were reported by other authors [38, 39].

As seen in Tables 3S–11S, the facultatively anaerobic *A. bogrovensis* was found to contain a total of 12 identified polar lipid classes with 172 molecular species, whereas the aerobic *A. rupiensis* contains six identified polar lipid classes with a total of 195 molecular species. Both species contain five classes of polar lipids that could not be identified. We shall deal in detail only with those Tables that are not given in Supplements.

Individual classes of separated phospholipids were identified by ESI–MS. Based on literature data [40, 41] and our previous results we were able to identify and quantify individual molecular species, see Tables 3–7. The only potential uncertainty concerned the identification of PtdGro

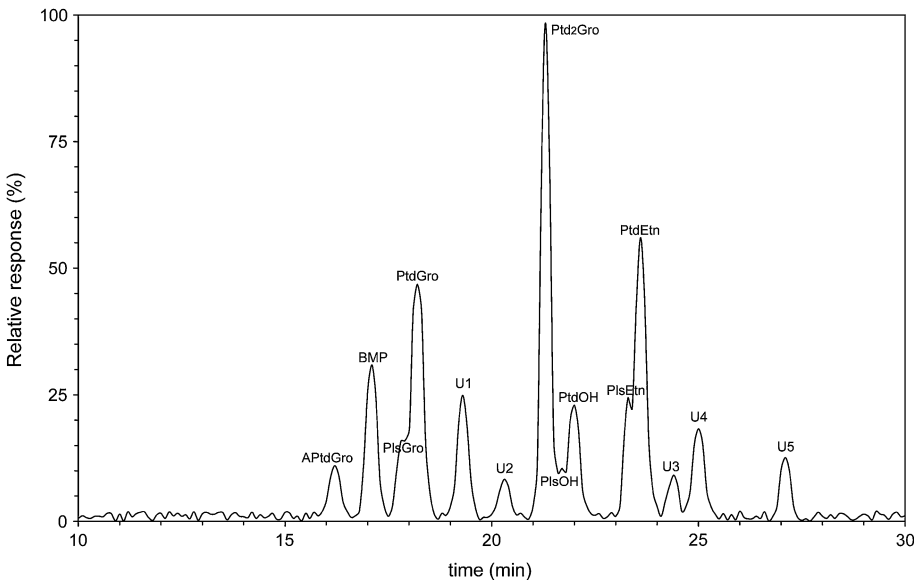

Fig. 1 LC/ESI chromatogram of the phospholipids from *A. bogrovensis*

Table 1 Fatty acid compositions (weight percentages) of *A. bogrovensis* and *A. rupiensis* as determined by GC–MS

Fatty acid	*A. bogrovensis*	*A rupiensis*
14:0[a]	0.3	0.2
i-15:0	22.9	26.6
ai-15:0	5.3	1.5
15:0	0.4	0.2
i-16:0	13.3	15.0
16:0	8.9	10.5
i-17:0	20.2	28.3
ai-17:0	21.5	4.6
17:0	0.3	0.2
i-18:0	0.5	0.2
18:0	6.4	12.6
20:0	tr	0.1
22:0	tr	nd

[a] First numeral, number of carbon atoms in the chain; second numeral, number of double bond; *i* isoacid; *ai* anteisoacid

tr traces (between 0.01 and 0.1 % of total FAs)

nd not detected (below 0.01 % of total FAs)

and BMP [26, 42]. However, the HILIC column provided a base-line separation without any problems.

Both phospholipids contain two acids, two glycerol moieties and one phosphodiester and the only difference between them is thus in the position of one of the acyls. For this reason the two phospholipids cannot be distinguished by mass spectrometry alone and it is necessary to use HPLC for their separation [26, 42].

Tandem mass spectrum of PtdGro 15:0/15:0 and BMP 15:0/15:0 in negative-ion mode produced similar fragmentation for both compounds, making specific determination of either lipid in the presence of the other difficult. Intense product ions were observed for the acyl (*m/z* 241) and for the glycerophosphate fragment at *m/z* 153. Minor differences in the intensities were observed for the ions at *m/z* 451 and 469 corresponding to the neutral loss of the $[M–H–ketene]^-$ and $[M–H–RCOOH]^-$. One clear distinction between PtdGro and BMP was the ability of PtdGro to lose the glycerol moiety without the loss of a fatty acid, i.e. a daughter ion of mass $[M–H–74]^-$ was always observed only for PtdGro but not for the compound with retention time 17.1 min. Thus, the neutral loss of 74 Da, i.e. ion at *m/z* 619, is specific for the 15:0/15:0 PtdGro species [43] and we concluded that this peak must contain BMP, for which it holds that each of the two glycerol moieties is esterified by one fatty acid.

Plasmenyl phospholipids were identified on the basis of our previously described fragmentation [28] and the method described by Hsu and Turk [44]. Briefly, the mass spectrum of all plasmenyl phospholipids is dominated by a set of RCO^-, $[M–H–RCOOH]^-$, $[M–H–R'CH=C=O]^-$, and $[M–H]^-$ ions that identify the fatty acid substituent. Ions reflecting the 1-*O*-alk-1′-enyl moiety are minor or not present but complete structural characterization of plasmenyl phospholipids can be carried out on the basis of the above ions.

Quantification of individual molecular species of the four classes of plasmalogens is given in Tables 3S–5S. Although the base-line separation of diacyl and alkenylacyl (plasmalogen) of the appropriate class of phospholipid was not successful, we succeeded in quantifying individual molecular species by using the method of Hsu and Turk [44]. Briefly, dissociation of the $[M–H–R_2COOH\text{-polar head group}]^-$ ions from the $[M–H]^-$ ions of plasmalogen phospholipid that have undergone consecutive losses of the fatty acid substituent at *sn*-2 and the polar head group readily gives the structural information about the radyl group at *sn*-1, resulting in semiquantification of appropriate molecular species. This is essentially a neutral loss scan with a mass offset that correlates with the mass of the specified neutral losses, i.e. fatty acid at *sn*-2 position and polar head group of the phospholipid.

Analysis of *A. rupiensis* lipids (Fig. 2) showed that the content of complex lipids is markedly different from that in *A. bogrovensis*. Based on previously published data [45] it can be assumed that the major peaks in the chromatogram are mainly PtdGro and Ptd_2Gro or their derivatives (Figs. 3, 4). As seen from the chromatogram, five other peaks could not be identified. The molecular species of nine identified classes of phospholipids are mentioned in Tables 12S–18S.

Only a few papers are devoted to the analysis of substituted PtdGro or Ptd_2Gro by mass spectrometry in Gram-positive bacteria. Peter-Katalinic and Fischer [20] fundamentally contributed to the identification of cardiolipin derivatives, i.e. Ptd_2Gro substituted on the *sn*-2 hydroxyl of the middle glycerol with α-D-glucopyranosyl- (gluco-Ptd_2Gro), D-alanyl- (alanyl-Ptd_2Gro), or L-lysyl- (lysyl-Ptd_2Gro) moieties. A review describing the presence of glucosyl-Ptd_2Gro, lysyl-Ptd_2Gro, alanyl-Ptd_2Gro, lysyl-PtdGro, etc. was published by Geiger et al. [11].

Analysis of PtdGro and Ptd_2Gro derivatives consisted in their separation on a HILIC column and identification by mass spectrometry. Mass spectra of derivatives of Ptd_2Gro have been described by, e.g., Peter-Katalinic and Fischer [20], who used positive and negative FAB to identify α-D-glucopyranosyl-, D-alanyl- and L-lysylcardiolipins. Negative ionization was more convenient in all cases since, due to the decreased formation of an alkali ion adduct, the negative-ion mass spectrum of the substituted-Ptd_2Gros provided a less complicated pattern than the positive-ion-mode and yielded sufficient information for elucidating the structure of most peaks in the chromatogram.

Based on literature data we assumed that all substituted derivatives of both PtdGro (Table 2) and Ptd_2Gro (Table 3)

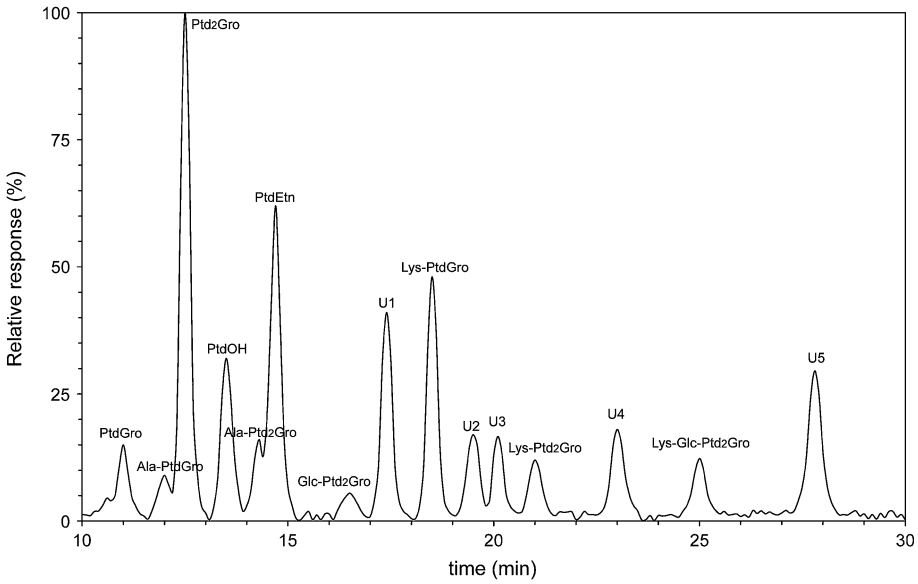

Fig. 2 LC/ESI chromatogram of the phospholipids from *A. rupiensis*

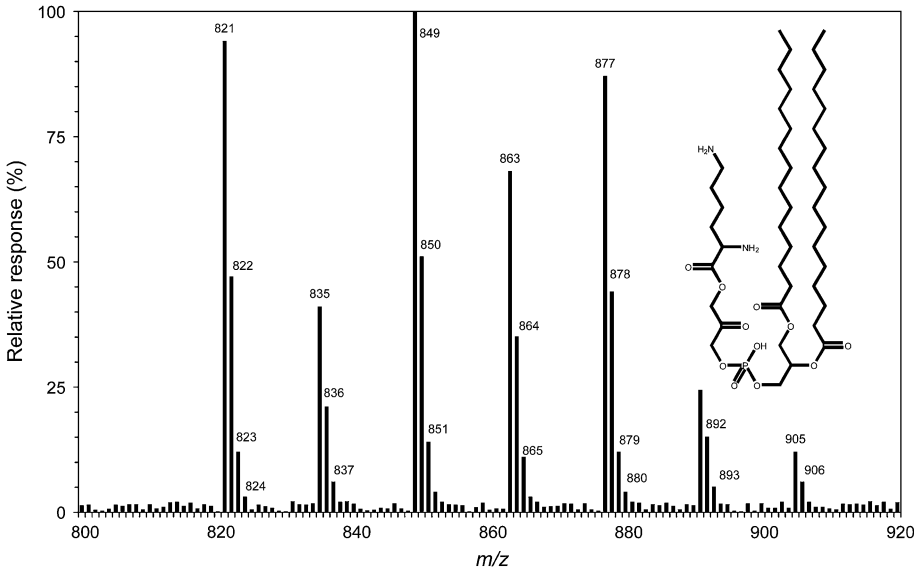

Fig. 3 ESI-mass spectrum of Lys-PtdGro from *A. rupiensis* in negative ion mode including structure of the major molecular species, i.e. 15:0/17:0-Lys-PtdGro having $[M–H]^-$ at 849 Da

will exhibit similar behavior, i.e. cleavage of a polar substituent—glucose or amino acid. For instance, the MS^2 product-ion spectrum of 17:0/15:0-lysyl-PtdGro (Fig. 5) shows $[M–H]^-$ ion at *m/z* 849, which was observed in the negative-ESI; this gives rise to the ion at *m/z* 721 arising from loss of a lysine (128 Da) from deprotonated ion of a substituted-PtdGro.

The MS/MS spectrum of alanyl-PtdGro (15:0/17:0-alanyl-PtdGro) also shows two ions. One of them, at *m/z* 88, corresponds to the anion of alanine while the other, at *m/z* 718, arises by neutral loss of 88 Da (alanine) from 15:0/17:0-alanyl-PtdGro ($[M–H]^-$ at *m/z* 806).

The splitting off of a neutral moiety will transform the unsubstituted PtdGro to a simpler structure and further fragmentation in mass spectrometer is thus identical with the fragmentation of unsubstituted PtdGro [46] described above.

The structure of lysyl-PtdGro was further confirmed by the presence of ion at *m/z* 647 originating from the loss of the dehydrated glycerol head group (74 Da), along with the ion at *m/z* 405, arising from *m/z* 479 by loss of dehydrated glycerol or from *m/z* 497 by loss of a glycerol residue (92 Da). The structure was further confirmed by the presence of the ions at *m/z* 269 and at *m/z* 241 corresponding to C17 and C15 saturated carboxylate anions, respectively. The ions at *m/z* 479 and *m/z* 497 arise from losses of the C15 acyls as an acid or a ketene, respectively, along with the ions at *m/z* 451 and *m/z* 469 corresponding to the analogous losses of C17 fatty acid moiety. Differentiation of *sn*-1 and *sn*-2 position was based on the abundance of

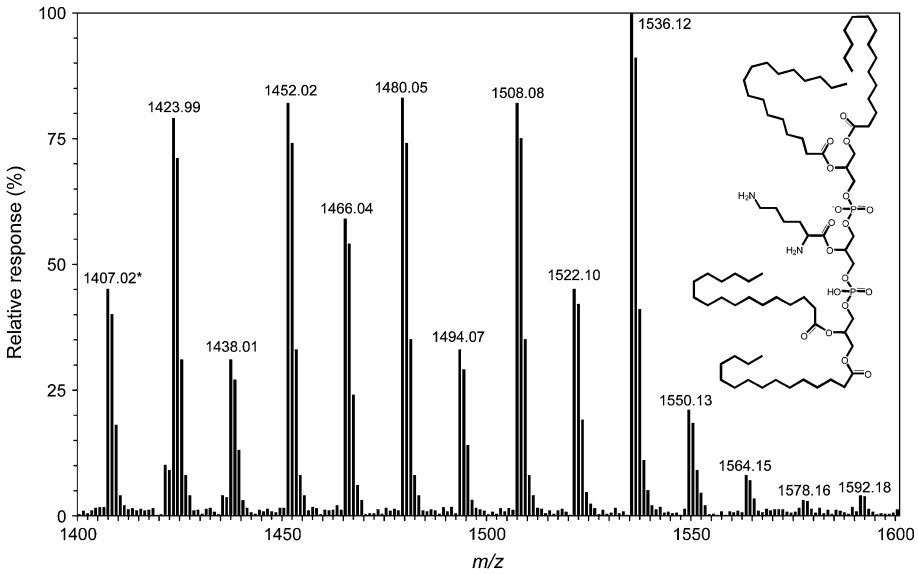

Fig. 4 ESI-mass spectrum of Lys-Ptd$_2$Gro from *A. rupiensis* in negative ion mode including structure of the major molecular species, i.e. 15/17/17/15-Lys-Ptd$_2$Gro having $[M–H]^-$ at 1,480.02 Da. *Asterisk* Ion at 1407.02 Da arises by splitting of lysine from $[M–H]^-$ at 1,536.12 Da

Table 2 Lys-PtdGro identified in *A. rupiensis*, the masses of protonated molecules ($[M–H]^-$), their ACN, and relative quantities of appropriate molecular species (%), including total %

$[M–H]^-$	ACN	Mol spec	%	Mol spec	%	Total %
821	30	15/15	21.9			21.9
835	31	15/16	9.6			9.6
849	32	15/17	19.7	16/16	3.5	23.2
863	33	15/18	7.5	16/17	8.2	15.7
877	34	16/18	3.1	17/17	17.0	20.1
891	35	17/18	6.7			6.7
905	36	18/18	2.8			2.8

two pairs of ions: those at *m/z* 479/497 were, respectively, more abundant than those at *m/z* 451/469; this documents that C17 FA is present in position *sn*-1 and C15 FA in position *sn*-2. Based on the above findings, the given molecular species can be determined as 17:0/15:0-lysyl-PtdGro. The situation was complicated by the presence of an isobaric molecule, i.e. 16:0/16:0-lysyl-PtdGro having characteristic ions at *m/z* 255, 391, 465, 483, etc., see also Fig. 5. Further aminoacyl-PtdGros or aminoacyl-Ptd$_2$Gros derivatives were identified in a similar way.

The major fragmentation pathway of Lys-Ptd$_2$Gro (Fig. 6) derivative gives an $[M–H]^-$ ion at *m/z* 1479, which is shown in Fig. 7. As noted above, both Lys-PtdGro and Lys-Ptd$_2$Gro undergo the neutral loss of a lysine moiety; the complex lipid (Lys-Ptd$_2$Gro) has thus in fact been translated into the simplified structure (Ptd$_2$Gro) and analyzed as previously described [27, 47], see also Fig. 7.

The structure of hexosyl-Ptd$_2$Gro was deduced on the basis of knowledge of negative mass spectra published previously by several authors [20, 47, 48].

The major derivative of hexosyl-Ptd$_2$Gro gives an $[M–H]^-$ ion at *m/z* 1514 and an $[M–2H]^{2-}$ ion at *m/z* 756. CID of the $[M–2H]^{2-}$ ions at *m/z* 756 at a collision energy of 50 eV yields two carboxylate anions at *m/z* 241 and *m/z* 269, reflecting the two fatty acyl moieties, i.e. both saturated C15 and C17 acids, in the molecule. As observed before in the detection of fragment ions due to the neutral loss of a hexose moiety, a fragmentation type was not detected in the MS/MS experiment using the $[M–2H + Na]^-$ as a precursor ion but only for $[M–H]^-$ [48]. The whole demonstration of hexosyl-Ptd$_2$Gro structure has thus in fact been translated into the simplified structure (Ptd$_2$Gro) and analyzed as previously described [47], see also above.

Table 3, which presents 29 molecular species of Lys-Ptd$_2$Gro, which is in agreement with the content of total FAs. A similar picture emerges in Table 2 describing the content of Lys-PtdGro, which also shows 15/15-, 17/17- and 15/17-Lys-PtdGro as major molecular species.

The peak with retention time 25.0 min (Fig. 2) is assumed to belong to glucosyl-Ptd$_2$Gro, in which some of the hydroxyls of glucose are esterified by an amino acid (lysine). Due to an insufficient amount of biomass we could not determine the complete structure and its full determination, as well as that of the as yet unknown peaks, will be the topic of our forthcoming study.

So far, not a single paper has appeared which would describe the separation and subsequent identification of any complex lipid having both straight- and branched-chain FAs in the molecule. In a previous study [28] we succeeded in separating synthetic triacylglycerols differing only in straight- and branched chain FAs (specifically 17:0/17:0/17:0 from ai-17:0/ai-17:0/ai-17:0). Unfortunately, because the mass spectra of both triacylglycerols are identical, the

Table 3 Lys-Ptd$_2$Gro identified in *A. rupiensis*, the masses of protonated molecules ([M–H]$^-$), their ACN, and relative quantities of appropriate molecular species (%), including total %

[M–H]$^-$	ACN	Mol spec	%	Mol spec	%	Mol spec	%	Mol spec	%	Mol spec	%	Total %
1423	60	15/15/15/15	12.6									12.6
1437	61	15/15/15/16	5.0									5.0
1451	62	15/15/16/16	2.0	15/15/15/17	11.0							13.0
1465	63	15/16/16/16	0.8	15/15/15/18	4.3	15/15/16/17	4.3					9.4
1479	64	15/17/17/15	12.5	15/16/16/17	0.7							13.2
1493	65	16/16/16/17	0.7	15/15/17/18	3.8	15/16/16/18	0.7					5.2
1507	66	15/17/17/17	8.3	16/16/17/17	1.5	16/16/16/18	0.3	15/15/18/18	1.5	15/16/17/18	1.5	13.1
1521	67	16/17/17/17	3.3	15/17/17/18	3.3	16/16/17/18	0.6					7.2
1535	68	17/17/17/17	14.4	16/17/17/18	1.3	16/16/18/18	0.2					15.9
1549	69	15/18/18/18	0.5	17/17/17/18	2.8							3.3
1563	70	17/17/18/18	1.1	16/18/18/18	0.2							1.3
1577	71	17/18/18/18	0.4									0.4
1591	72	18/18/18/18	0.6									0.6

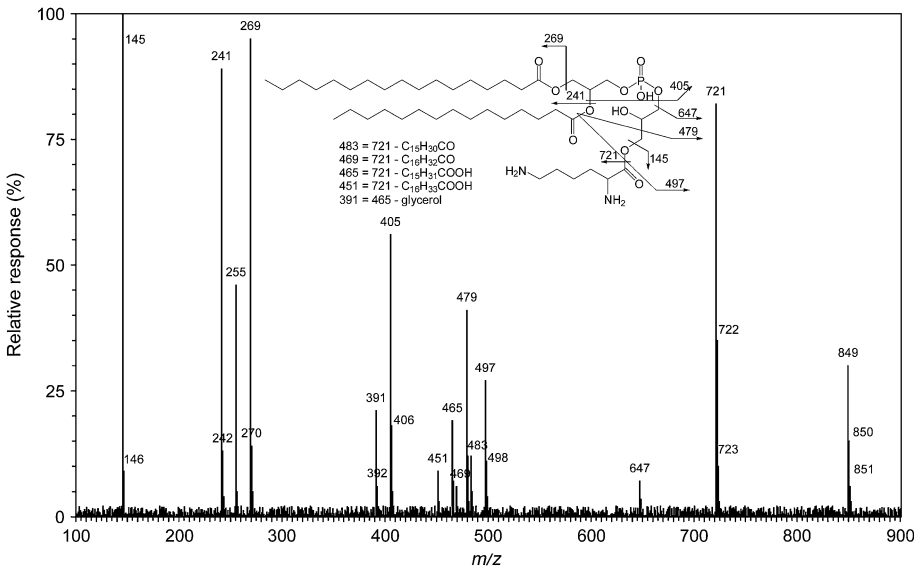

Fig. 5 The tandem ESI product-ion spectrum of the [M–H]$^-$ ion of 16/16-Lys-PtdGro and 17/15-Lys-PtdGro including fragmentation

individual molecular species shown in all tables and figures can contain straight- and branched-chain FAs. The only exception is the separation and identification of straight- and branched-chain FAs as methyl esters by GC–MS (Table 2), which presented no problems.

In this study we demonstrated that the ability of one species, i.e. *A. bogrovensis* to grow under facultatively anaerobic conditions quite clearly affects both qualitative and quantitative composition of lipids. The majority of lipids in the two strains under study was qualitatively the same but the strain of *A. rupiensis* cultivated under aerobic conditions showed a much wider range of polar lipids, i.e. aminoacyl-phospholipids, including as yet undescribed amino-phospho-glyco-lipid. In addition, to our knowledge we are the first to determine plasmalogens in a facultatively anaerobic bacterium. The recent review about plasmalogens [11] still refers to this as unproven.

We believe that the ability to synthesize many different lipids must also be seen in a taxonomic and evolutionary context of classification of this genus. Our study points to the value of chemotaxonomy within the bacilli and to the need for including such analyses in all future taxonomic work on this and other groups [49].

Experimental

The standard lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (16:0, 18:1 PtdGro) used in the experiments to verify the bacterial compound structure was purchased from Avanti Polar Lipids (Alabaster, AL, USA).

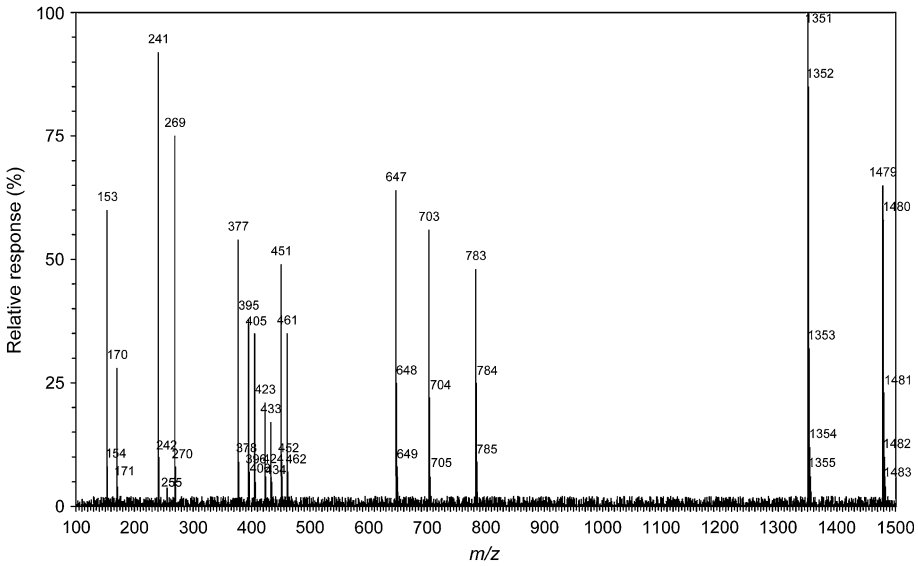

Fig. 6 The tandem ESI product-ion spectrum of the $[M–H]^-$ ion of 15/17/17/15-Lys-Ptd$_2$Gro and 15/16/16/17-Lys-Ptd$_2$Gro

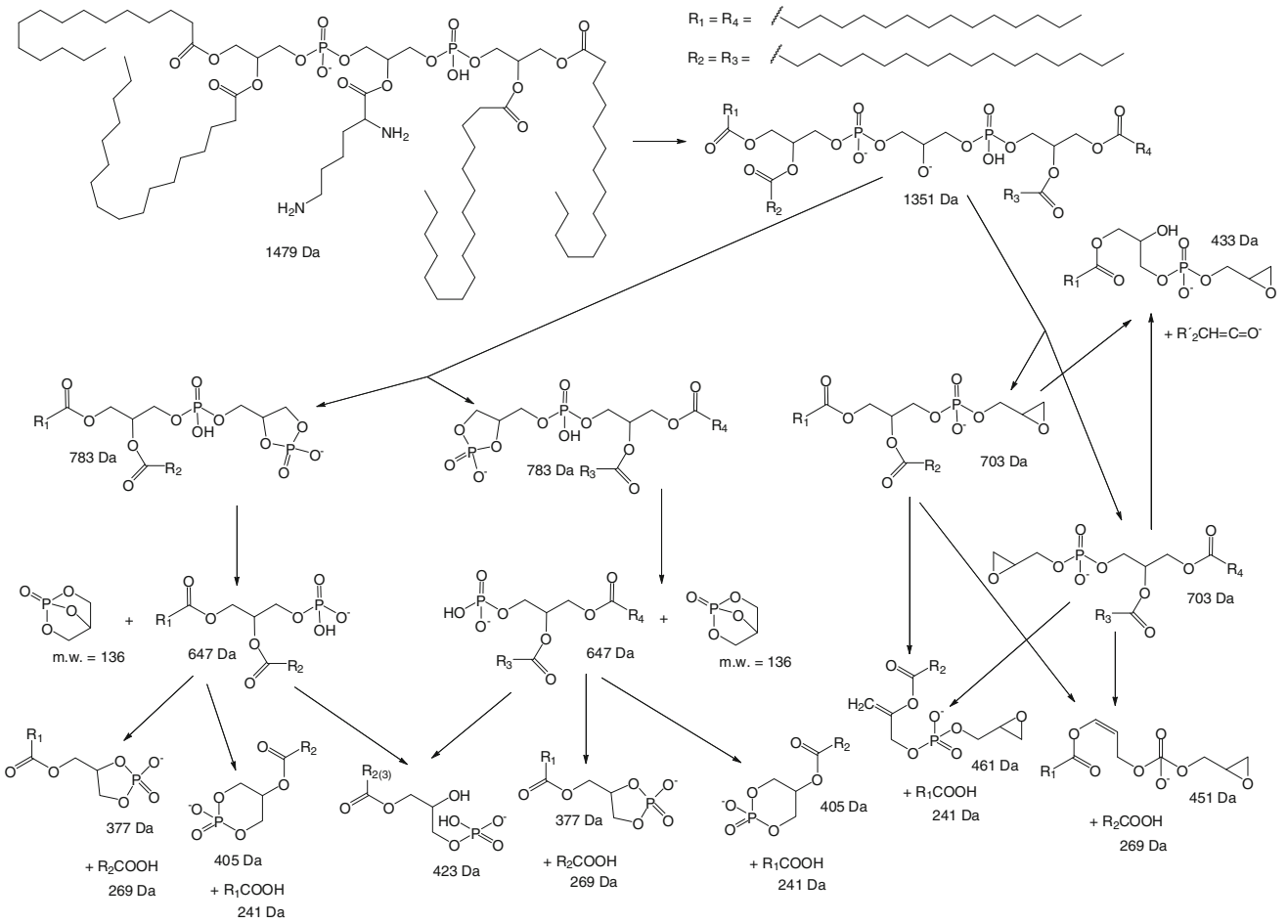

Fig. 7 The major fragmentation pathways proposed for formation of ions from the $[M–H]^-$ ion of lysyl-cardiolipin (15/17/17/15-Lys-Ptd$_2$Gro)

Both strains were cultivated in the medium containing 0.2 % peptone and 0.1 % yeast extract on a bath shaker at 240 rpm for 18 h at their optimal pH and temperature. *Anoxybacillus rupiensis* was cultivated at pH 6.5 and 55 °C; *A. bogrovensis* was cultivated at pH 8.0 and 65 °C under anaerobic conditions, i.e. in the absence of

atmospheric oxygen. Cells were harvested after centrifugation (4,000 rpm for 20 min) and two washes with distilled water, and lyophilized. The dry biomass after lyophilization of both strains was 0.367 g/L for *A. rupiensis* and 0.233 g/L media for *A. bogrovensis*.

The extraction procedure was based on the method of Bligh and Dyer [29], except that 2-propanol was substituted for methanol, since isopropanol does not serve as a substrate for phospholipases [50]. The alcohol-water mixture was cooled, one part chloroform was added and the lipids were extracted for 30 min. Insoluble material was sedimented by centrifugation and the supernatant was separated into two phases. The aqueous phase was aspirated off and the chloroform phase was washed three times with two parts 1 M KCl each. The resulting chloroform phase was evaporated to dryness under reduced pressure.

First, total lipid extracts were applied to Sep-Pak Cartridge Vac 35 cc (Waters; with 10 g of aminopropylsilica-based polar bonded phase), and from the cartridge were subsequently eluted nonacidic lipids with 40 mL of chloroform–methanol (7:3), and acidic lipids with 30 mL of chloroform–methanol–concentrated aqueous ammonia (70:30:2) containing 0.4 % (w/v) ammonium acetate. The eluate was reduced in volume and subjected to LC–MS.

HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Hewlett Packard, USA), and two Ascentis® Express HILIC HPLC columns [2.7 µm particle size, L × I.D. 15 cm × 2.1 mm (Supelco, Prague)] connected in series. LC was performed at a flow rate of 0.45 mL/min for the phospholipids from the *A. bogrovensis* and 0.58 mL/min for the phospholipids from the *A. rupiensis*, respectively, with a linear gradient from mobile phase containing methanol/acetonitrile/aqueous 1 mM ammonium acetate (50:30:20, v/v/v) to methanol/acetonitrile/aqueous 1 mM ammonium acetate (10:70:20, v/v/v) for 60 min. The whole HPLC flow was introduced into the ESI source without any splitting.

The detector was an Applied Biosystems Sciex API 4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) using electrospray mass spectra. The ionization mode was negative, the nebulizing gas (N_2) pressure was 350 kPa and the drying gas (N_2) flow and temperature were 9.2 L/min and 310 °C, respectively. The electrospray needle was at ground potential, whereas the capillary tension was held at 4,000 V. The cone voltage was kept at 250 V. The mass resolution was 0.1 Da and the peak width was set to 5 s. For an analysis, total ion currents (full scan) were acquired from 80 to 2,000 Da.

Mass spectra of CID ions were acquired by colliding the Q1 selected precursor ions with Ar gas at a collision target gas and applying collision energy of 50 eV in Q2. Scanning range of Q3 was *m/z* 80–2,000 with a step size of *m/z* 0.25 and a dwell time of 1 ms. A peak threshold of 0.2 % intensity was applied to the mass spectra. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.4.1 software.

Gas chromatography–mass spectrometry of FAME was done on a GC–MS system consisting of Varian 450-GC, Varian 240-MS ion trap detector with external ionization (EI), and CombiPal autosampler (CTC, USA). The sample was injected onto a 25 m × 0.25 mm × 0.1 µm Ultra-1 capillary column (Supelco, Czech Republic) under a temperature program: 5 min at 50 °C, increasing at 10 °C min^{-1} to 320 °C and 15 min at 320 °C. Helium was the carrier gas at a flow of 0.52 mL min^{-1}. All spectra were scanned within the range *m/z* 50–600.

Comparison of retention times of commercially obtained standards [Bacterial Acid Methyl Ester (BAME) Mix (Supelco, Prague)] and previously published results [51] were used for identification.

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